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Cytogenomic Analyses Reveal the Structural Plasticity of the Chloroplast Genome in Higher Plants

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A DNA fiber-based fluorescence in situ hybridization (fiber-FISH) technique was developed to analyze the structure and organization of a large number of intact chloroplast DNA (cpDNA) molecules from Arabidopsis, tobacco, and pea. Using this cytogenomic approach, we determined that 25 to 45% of the cpDNA within developing leaf tissue consists of circular molecules. Both linear and circular DNA fibers with one to four copies of the chloroplast genome were present, with monomers being the predominant structure. Arabidopsis and tobacco chloroplasts contained previously unidentified multimers (>900 kb) consisting of six to 10 genome equivalents. We further discovered rearranged cpDNA molecules of incomplete genome equivalents, confirmed by both differential hybridizations and size estimations. The unique cpDNA organization and novel structures revealed in this study demonstrate that higher plant cpDNA is more structurally plastic than previous sequence and electrophoretic analyses have suggested. Additionally, we demonstrate how the fiber-FISH–based cytogenomic approach allows for powerful analysis of very rare events that cannot be detected by traditional techniques such as DNA gel blot hybridization or polymerase chain reaction.

INTRODUCTION

The chloroplasts of higher plants possess small, self-replicating DNA molecules varying in size from 120 to 220 kb with highly conserved gene content across species (Palmer, 1992). Restriction enzyme site mapping of the chloroplast genome in many plants predicted a circular molecule with two large inverted repeats (IRs) (reviewed in Palmer, 1992). Electron microscopy (EM) and pulsed-field gel electrophoresis (PFGE) have been used to investigate chloroplast DNA (cpDNA) conformation and copy number. EM studies revealed only monomers and a low percentage of dimer molecules from CsCl-isolated cpDNA (Kolodner and Tewari, 1975a). The advent of PFGE allowed for analysis of cpDNA by embedding the plastids in agarose to reduce DNA breakage or degradation (Deng et al., 1989; Backert et al., 1995). PFGE analysis of spinach and tobacco revealed a low percentage of multimeric cpDNA forms, with none greater than tetrameric size (Deng et al., 1989; Backert et al., 1995).

Bendich and Smith (1990) used PFGE to separate cpDNA, excised the discrete bands, and analyzed the ethidium bromide–stained cpDNA within the bands via UV light microscopy. The data showed that watermelon and pea chloroplasts possess linear oligomeric molecules of various sizes. Circular molecules were observed when the molecules remaining at or near the loading wells were analyzed. Bendich (1991) also analyzed cpDNA by ethidium bromide staining of DNA from individual lysed chloroplasts. It was estimated that 20 to 80% of the cpDNA within watermelon and pea exists in circular forms (Bendich, 1991).

All previous reports of cpDNA structure described a predominance of monomeric circular molecules, with higher order multimeric linear conformations present at lower frequencies (Deng et al., 1989; Bendich and Smith, 1990; Backert et al., 1995). However, the exact quantification of different structures in a population of cpDNA molecules has not been determined. In addition, there have been no reports of molecules of greater than tetrameric size or cpDNA structures that deviate from multimers of genome equivalent size. The lack of such data could be attributed to the methods used in these studies. Some techniques require ultracentrifugation to purify the DNA and may lead to artifacts during specimen preparation (Bendich, 1991). It is also difficult to observe individual molecules across a population using the previously described techniques. EM allows for analysis of individual DNA molecules, but it is difficult and labor intensive to observe hundreds or thousands of such molecules. The extraction of DNA bands after PFGE reduces variability among samples due to the uniformity of PFGE fractions. This is primarily a population-based approach, and the diversity of complex structures remains difficult to resolve.

To visualize the intact structure of different types of DNA molecules (circular, linear, and more complex forms) in

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organelles, a technique must not damage DNA structure as it exists in the organelle or contribute to artifacts. Recently developed in situ hybridization techniques based on extended DNA fibers allow visual analysis of large DNA molecules using light microscopy (Fransz et al., 1996; Jackson et al., 1998). Jackson et al. (1999) demonstrated that intact circular DNA of bacterial artificial chromosomes can be visualized using the DNA fiber-based fluorescence in situ hybridization (fiber-FISH) procedure. We developed a fiber-FISH–based methodology to analyze intact cpDNA molecules from Arabidopsis, tobacco, and pea. This cytology-based molecular analysis of a complete genome (cytogenomics) revealed that the chloroplast genome is highly plastic and exists as a heterogeneous mixture of sizes and physical conformations not identified previously. Novel applications of cytogenomics to organellar studies are discussed.

RESULTS

PFGE Analysis of cpDNA from Tobacco and Pea

PFGE was used to estimate the sizes of cpDNA molecules present in our preparations of chloroplasts from pea and tobacco. Hybridization of the *rbc*L gene probe to DNA gel blots from PFGE revealed four and two prominent bands from the tobacco and pea preparations, respectively (Figure 1). Some DNA did not migrate out of the well or stayed in the compression zone, suggesting the existence of very large molecules. Significant hybridization signals were observed below the monomeric band, which may be due to both sheared cpDNA and possibly molecules with subgenomic sizes (see below). Under our PFGE conditions, large (>200 kb) circular or branching molecules would not be resolved as discrete bands, and our size estimations were based on the migration distances of linear molecules (Bendich, 1991). The lack of higher order (trimer and tetramer) bands in the pea preparation agrees with earlier work by Bendich (1991) and sug-

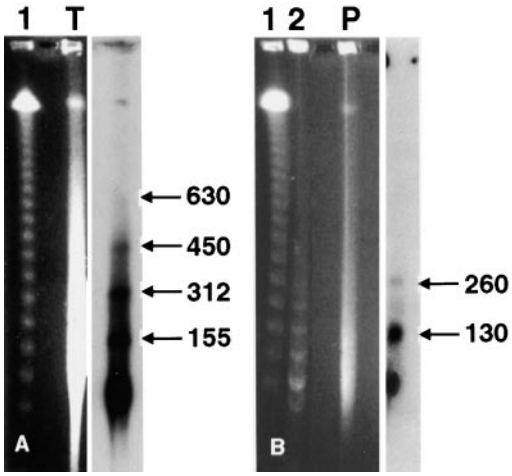

Figure 1. PFGE Analysis of Chloroplast DNAs from Tobacco and Pea.

(A) Tobacco.
(B) Pea.
Ethidium bromide–stained pulsed-field gels with a λ marker in lanes 1 (**[A]** and **[B]**) and a low-range PFGE marker (New England Biolabs, Beverly, MA) in lane 2 **(B)**. T and P indicate lanes containing embedded chloroplasts from tobacco and pea, respectively. Arrows designate the most prominent bands representing monomer (155), dimer (312), trimer (450), and tetramer (630) fragments in tobacco and monomer (130) and dimer (260) fragments in pea (in kilobases). PFGE conditions were 5 to 120 sec of pulse time at 4.5 V/cm, with a total run time of 46 hr.

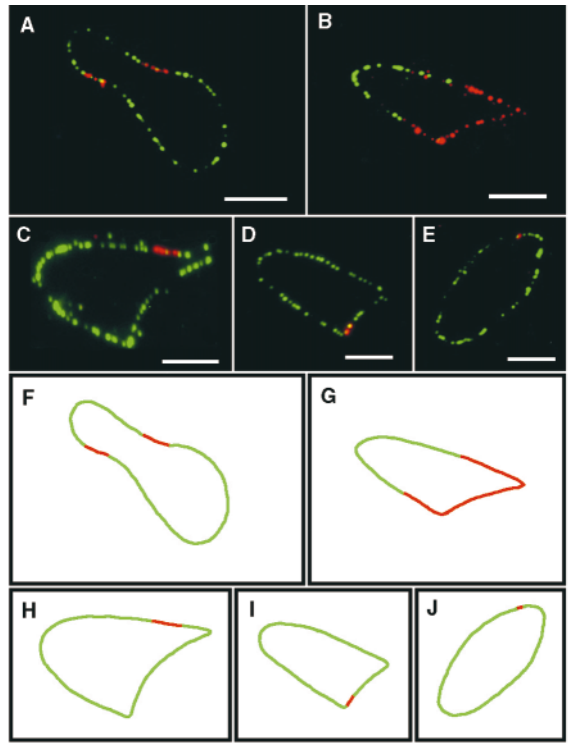

Figure 2. Fiber-FISH Images of Tobacco Chloroplast DNA.

(A) A monomeric circular cpDNA molecule. The green signal represents the entire cpDNA genome, and the red signal represents the 12.2-kb IR.
(B) A monomeric circular cpDNA molecule with ~87 kb (24 μm) of the green signal representing the LSC and 68 kb (17 μm) of the red signal homologous to both the SSC and IR regions.
(C) to **(E)** Three monomeric cpDNA molecules (green) together with hybridization signals (red) derived from the 12.5- **(C)**, 4.6- **(D)**, and 1.8-kb **(E)** cpDNA probes.
(F) to **(J)** Schematic illustrations of the cpDNA images, corresponding to **(A)** to **(E)**, respectively. Green and red lines represent the differential hybridization.
Bars = 5 μm.

gests that the pea chloroplast genome is less plastic, possibly due to the lack of IRs (Palmer and Thompson, 1982).

Fiber-FISH Visualizes Intact cpDNA Molecules at High Resolution

cpDNA was visualized using a modified fiber-FISH procedure (see Methods). We used the tobacco cpDNA, which has been sequenced completely (Shinozaki et al., 1986), to calibrate the accuracy of this technique. A 12.2-kb probe homologous to sequences within the IR (22 kb) region was used as a reference marker to identify intact monomeric circular cpDNA molecules (Figure 2A). We measured 50 random circular cpDNA fibers possessing two IR hybridization signals. From these measurements, we estimated the relationship between cytological distance and molecular size to be 1.0 kb = 0.26 ± 0.03 μm. Because of potential variation in DNA fiber extension, we had to determine the confidence level of our calibration across many molecules of various sizes. We measured the large single copy (LSC) region (87 kb), the small single copy (SSC) region together with the two IRs (68 kb), the SSC alone (25 kb), and the IR from 50 monomers. We also included an additional 72 random circular molecules possessing two or four IR signals representing monomer (156 kb) and dimer (312 kb) conformations. Regression analysis for the expected lengths against the measured average lengths for each of these classes (Table 1) resulted in a highly significant ($P < 0.001$) relationship ($r^2 = 0.998$) that was consistent with our initial estimates. Calibration was further confirmed by differential labeling of the LSC with one color and the SSC plus IRs with another color. Figure 2B shows a typical monomeric circular molecule with the expected sizes of 23.7 ± 4.6 μm of LSC and 17.4 ± 4.0 μm of SSC plus IR. With a reliable kilobase-to-micrometer relationship, it was possible to use both the IR and LSC/SSC probes to quantify the genome copy number (monomer, dimer, etc.) and the respective sizes of these units among different cpDNA conformations.

The resolution limit of our fiber-FISH technique was determined using three different probes from the LSC region. Probes of 1.8 kb (P18), 4.6 kb (P16), and 12.5 kb (P10) were hybridized to both tobacco and pea cpDNA to determine the detection sensitivity (Figures 2C to 2E). The 12.5-kb probe was consistently visible on nearly all DNA fibers. The signals derived from the 4.6-kb probe were also visible but with less intensity. However, the 1.8-kb probe was detected consistently only with two additional layers of fluorescent antibodies.

Various Genomic Conformations Are Present within Chloroplasts

Fiber-FISH allowed for detailed studies of the structure and organization of many individual cpDNA molecules (Figures 3A and 3B). Table 1 summarizes the classification of all molecules analyzed in this study. In Arabidopsis and tobacco, 42 and 45%, respectively, of the total cpDNA fibers were circular molecules. Monomeric circular molecules constitute 55% of the circular molecules and 26% of all cpDNA fibers in tobacco. The remaining 45% of circular molecules are multimers of different sizes present at decreasing frequencies. The metric distribution of circular molecules from tobacco is summarized in Table 2. The sizes of some molecules were significantly greater than expected based on PFGE and have not been documented previously. Approximately 17% of the fibers from Arabidopsis and tobacco were not classified (Table 1). It is not known if these tangled fibers are artifacts or biologically significant molecules.

Table 1. Frequency of Different cpDNA Structures across All Experiments in Three Species

	No. of Observations		
Structure[a]	Arabidopsis	Tobacco	Pea
Circular	126 (42%)	524 (45%)	59 (25%)
Linear	68 (23%)	250 (22%)	85 (36%)
Bubble/D-loop	25 (8%)	67 (6%)	5 (2%)
Lassolike	34 (11%)	115 (10%)	21 (9%)
Unclassified[b]	44 (16%)	203 (17%)	66 (28%)

[a] Each classification represents all molecules of that type regardless of size.
[b] DNA fibers that were coiled or folded and could not be classified

The number of IR hybridization signals can be used to classify circular molecules into discrete classes (Figure 3C). The graph presented in Figure 4 shows that the majority of analyzed circular molecules fell into the monomer or multimeric molecule class within the 95% confidence interval of our measurements. The monomer, dimer, trimer, and tetramer circular molecules were detected at decreasing levels (Table 2). There were also circular molecules that did not fall into these discrete classes (see below). Intact circular cpDNA fibers >600 kb and containing at least four copies of the genome were observed frequently. The single largest circular tobacco cpDNA fiber observed was >250 μm, resulting in a circular molecule of at least hexameric genome size. These data are similar to the PFGE results; however, a greater presence of larger (tetrameric and higher) molecules was observed under the microscope, possibly due to the resolution limits of circular molecules within a pulsed-field gel.

Linear fibers accounted for ~22% of the total chloroplast molecules in Arabidopsis and tobacco (Table 1). However, this may be an over estimation because some of the linear fibers are probably broken circles. Long linear fibers were observed consistently in all experiments (Figure 3D). The longest linear cpDNA fiber was ~1200 kb, a possible octomer, from tobacco (image not shown). These molecules

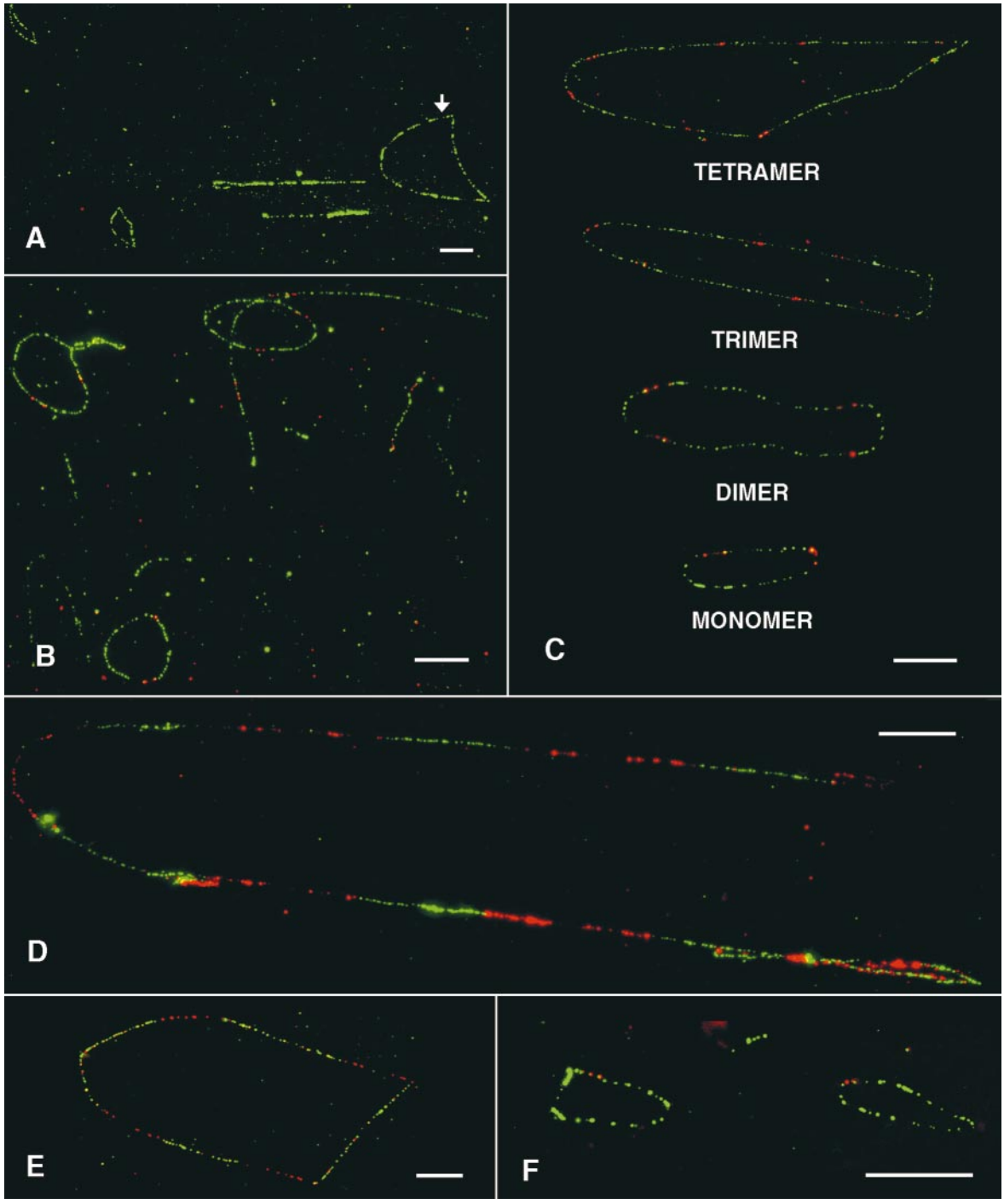

Figure 3. Structural Plasticity of cpDNA Molecules from Tobacco, Arabidopsis, and Pea.

(A) A wide-field view (magnification ×400) showing three circular cpDNA molecules from tobacco. The two small circles are monomer size, and the larger molecule is trimer size (arrow).
(B) A field of view (magnification ×630) showing tobacco chloroplast fibers hybridized with an IR probe (red FISH signal).
(C) Selected monomeric and multimeric cpDNA molecules from tobacco. Red signals are from the 12.2-kb IR probe.
(D) A complete linear hexameric tobacco molecule. The green signals represent LSC; the red signals show SSC and IR regions.
(E) A circular tetrameric chloroplast molecule from Arabidopsis. The green signals were derived from P1 clones MAB17 and MCI3, and the red signals are from P1 clone MAH2 (Sato et al., 1999).
(F) Two monomeric circular molecules from pea probed with a single-copy sequence possessing the origin of replication (red).
Bars = 10 μm.

cannot be classified into discrete classes because it is difficult to distinguish between true linear molecules and broken circles.

A similar diversity of cpDNA structures was observed in Arabidopsis. This chloroplast genome has been sequenced completely (154,478 bp) and contains the usual pair of IRs (Sato et al., 1999). Three overlapping P1 clones (MAB17, MAH2, and MCI3) spanning the entire chloroplast genome were used in fiber-FISH experiments. Figure 3E shows a head-to-tail tetrameric cpDNA molecule present within Arabidopsis chloroplasts. Both tobacco and Arabidopsis possessed similar frequencies of monomer and multimer conformations, with very similar structures. In both species, all multimeric molecules observed via differential hybridization signals existed as head-to-tail concatamers.

cpDNA Molecules from Pea Showed Less Structural Diversity Than Did Those from Tobacco and Arabidopsis

To further investigate the structure of chloroplast genomes among higher plants, we used plastids from garden pea in our cytogenomic analyses. Pea is a member of the Fabaceae and possesses a smaller chloroplast genome (~120 kb) with only a single copy of the sequence corresponding to the IRs (Palmer and Thompson, 1981). Extended cpDNA fibers of pea were hybridized with both the LSC/SSC probes and the 12.2-kb IR probe. Pea exhibited fewer circular molecules (25%) compared with tobacco and Arabidopsis but a correspondingly higher percentage (36%) of linear fibers (Table 1). As expected, the cpDNA from pea showed only one copy of the IR (Figure 3F). Pea exhibited fewer higher order multimers; no circular molecules larger than dimer size were revealed by fiber-FISH or PFGE (Figure 1). Across 236 pea cpDNA fibers, only two circular fibers were identified as significantly smaller than the monomeric size.

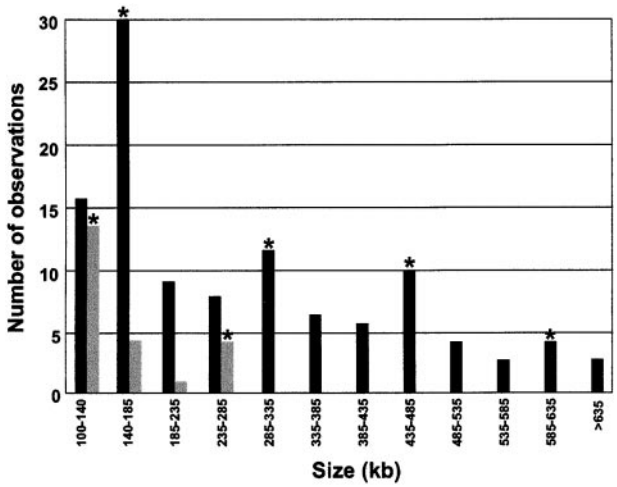

Figure 4. Size Variation of Circular Chloroplast DNA Molecules.

Black bars represent tobacco and gray bars represent pea molecules. Sizes were converted to kilobases based on 1.0 kb equaling 0.26 ± 0.03 μm. Asterisks represent the monomeric, dimeric, trimeric, and tetrameric molecules within a 99% confidence interval around the mean.

Table 2. Sizes and Distributions of Circular cpDNA Molecules Observed in Tobacco[a]

	Circumference[b]	LSC	SSC + IRs	IR
Range (μm)	24.4–53.5	12.3–27.8	9.6–19.3	1.9–4.1
Average size (μm)	40.2	21.8	15.7	3.0
Standard deviation	6.2	4.6	4.0	0.5
Calibrated size[c] (μm)	41.2	23.7	17.4	3.2
Size Distribution of Circular Molecules[d]	Average Size (μm)	Number	Percentage	
Monomer	40.2	55	55	
Dimer	82.4	17	17	
Trimer	123.8	10	10	
Tetramer	160.4	7	7	
Pentamer	202.1	5	5	
Hexamer	NA[e]	1	1	
Unclassified[f]	NA	5	5	

[a] LSC, large single-copy unit (90 kb); SSC, small single-copy unit (66 kb); IR, inverted repeat probe (12.2 kb).
[b] Observations of 50 molecules based on measuring circular molecules with a single pair of IR across two chloroplast preparations and 10 slides.
[c] Calibration based on regression analysis.
[d] Distribution of 100 circular molecules in tobacco across eight slides. Metric classifications are based on the number of hybridization signals obtained from a 36.6-kb single-copy probe.
[e] NA, not available; these molecules cannot be measured.
[f] Circular molecules that did not fit into the defined categories and were outside the 95% confidence interval.

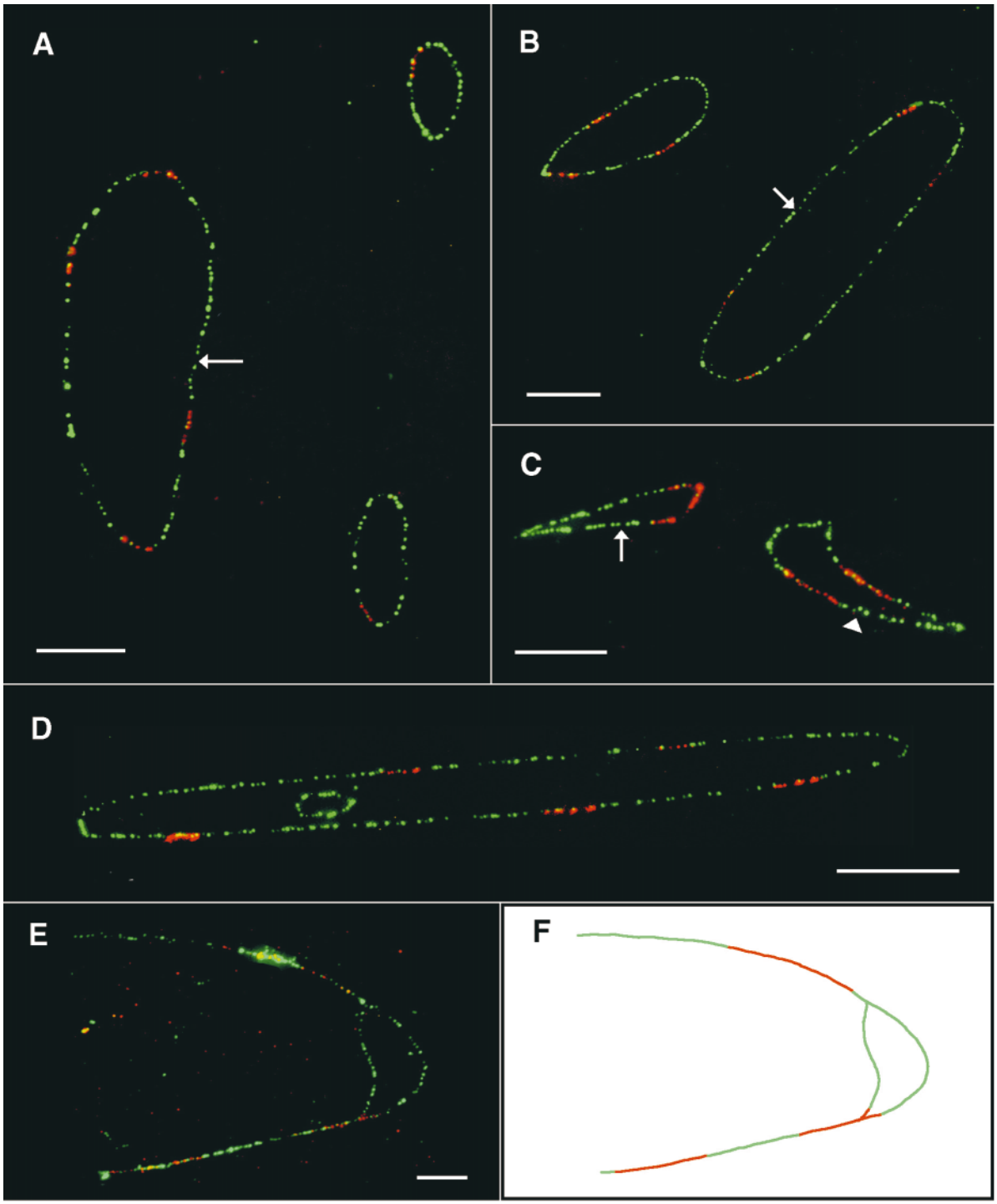

Figure 5. Atypical cpDNA Molecules.

(A) A typical head-to-tail dimer (arrow) from tobacco showing the 12.2-kb IR (red signals) and two small (133 and 110 kb) circular molecules exhibiting only a single IR.
(B) A typical head-to-tail dimer (arrow) from tobacco showing the IR (red signals). A smaller circular molecule (205 kb) exhibits three IRs.
(C) Two Arabidopsis cpDNA molecules hybridized with three probes homologous with 40.4 kb of the LSC region (red signal). The arrow points to a typical monomeric structure, and the arrowhead points to an ~190-kb molecule that has two hybridization signals.

Atypical cpDNA Molecules

The majority of the circular cpDNA molecules in tobacco and Arabidopsis were monomers or multimers; however, molecules of clearly different sizes were observed frequently. The FISH signals derived from the IR or other cpDNA probes served as landmarks to judge the structural variability of these molecules. We observed molecules without IRs (Figure 5D) or with one (Figure 5A), three (Figure 5B), and five (Figure 5D) repeats in tobacco and Arabidopsis. In some molecules, the distances between two IRs clearly deviated from the expected length. Some smaller molecules revealed by fiber-FISH may correspond to the signals below the monomer size in autoradiograms from PFGE DNA gel blots. We estimated that atypical molecules represented between 2 and 5% of total circular molecules (0.8 to 2% of all DNAs) in tobacco chloroplast. However, this number may be underestimated because we classified molecules as being atypical only when the number of hybridization signals deviated from the expected numbers and/or the linear distance between the signals was outside the 95% confidence interval of expected sizes.

Electrophoresis and in vitro assays suggested that cpDNA replication is initiated by a dual displacement loop (D-loop) formation, resulting in a Cairns forked structure (Kolodner and Tewari, 1975b). Replication continues by a rolling circle mechanism, possibly occurring at the end of the replication cycle (reviewed in Heinhorst and Cannon, 1997). We frequently observed circular plastid molecules with one tail (lassolike fibers) or two tails (bubble- or D-loop-like fibers) in independent preparations (Table 1; Lilly, 2000). Initially, these complex forms were thought to be artifacts; however, these structures were detected consistently in all hybridization experiments. Thus, some of these structures may be replication intermediates. Figure 5E shows a DNA molecule with morphology similar to the D-loop mode of DNA replication (Kolodner and Tewari, 1975b; Kunnimalaiyann and Nielsen, 1997).

DISCUSSION

A number of different techniques have been used to study cpDNA structure. Early methods, including EM and reassociation kinetics, revealed single circular cpDNA molecules of 130 to 160 kb, with occasional dimeric configurations and replication intermediates (Kolodner and Tewari, 1975a, 1975b). Restriction enzyme site mapping across a plethora of plants demonstrated that the cpDNA has a circular plasmidlike structure, with the only significant difference being the number of IR regions (reviewed in Palmer, 1992). Restriction enzyme site mapping has the advantage over EM and reassociation kinetics in that smaller DNA fragments can be analyzed, resulting in less error, and multiple restriction enzymes can be used to map the same molecule (Palmer and Thompson, 1981). A more recently used technique was PFGE of chloroplasts embedded in agarose (Deng et al., 1989; Backert et al., 1995), which eliminated the need to purify cpDNA and reduced shearing. However, the multimer-sized PFGE bands were not believed to contain circular molecules. Based on visualization of the excised fragments from PFGE via moving picture microscopy, Bendich and Smith (1990) and Bendich (1991) proposed that circular cpDNA molecules do not migrate in the prominent PFGE bands because such molecules may become hooked on agarose fibers and the secondary structures limit movement through the gel matrix.

We used a fiber-FISH method to develop a cytogenomic approach for the analysis of cpDNA. This cytogenomic approach offers several advantages over earlier techniques. (1) This methodology allows for reliable analysis of nearly all of the molecules released from a lysed chloroplast. (2) Many individual intact molecules can be analyzed in a relatively short time, and data from a large number of molecules allow quantification of the percentages of each type of structure in the population of cpDNA molecules. (3) DNA fragments from different parts of the cpDNA can be labeled and mapped. This flexibility significantly increases the power to analyze individual molecules and to reveal structural variability that would not be revealed by earlier methods.

Cytogenomic investigations of the cpDNA of tobacco and Arabidopsis revealed the previously undocumented structural plasticity of the chloroplast genome. Both tobacco and Arabidopsis possess the usual chloroplast genome of ~155 kb with IRs (Shinozaki et al., 1986; Sato et al., 1999). We found that circular molecules accounted for 45% of the total cpDNA in these two species. This is within the estimate given by Bendich and Smith (1990) of 20 to 80% based on analysis by PFGE and moving pictures. Monomers were the

Figure 5. (continued).

(D) An ~660-kb cpDNA molecule from tobacco exhibiting five IRs (red signals). A significantly smaller (~40 kb) DNA molecule shows no hybridization to the IR probe.

(E) A putative D-loop-like molecule from Arabidopsis. The red signals are derived from P1 clone MAH2 that covers the IRs, SSC, and part of the LSC regions (Sato et al., 1999).

(F) A schematic illustration of the image shown in **(E)**.

Bars = 10 μm.

most predominant form, accounting for ~60% of the circular molecules within the tobacco chloroplasts (Table 2). Most multimeric molecules were head-to-tail concatamers. Circular and linear molecules up to 800 and 1000 kb, respectively, were observed on most preparations. These massive multimeric molecules may be the result of recombination and/or DNA replication (Deng et al., 1989; Bendich and Smith, 1990; Backert et al., 1995). It is not known whether these massive higher order molecules are functional or if they are later cleaved into smaller units.

Autoradiograms from PFGE and gel blot hybridizations of cpDNA revealed faint signals between major bands (Figure 1) (Deng et al., 1989; Backert et al., 1995). These faint signals may represent sheared DNA derived from the multimeric molecules. An alternative explanation is that the signals between the major bands in PFGE represent true size variations present within the chloroplast. Our cytogenomic analysis of cpDNA revealed major structural changes representing partial genome units that have not been identified previously. Atypical cpDNA molecules were classified unambiguously based on the numbers of hybridization signals from known probes (Figure 5). These rare atypical molecules were observed for both monomeric and multimeric molecules. Most size variants fell between the monomer–dimer groups (Figure 4). We propose that the faint hybridization signals seen on PFGE autoradiograms of cpDNA represent a combination of both sheared DNA and low levels (0.8 to 2% of all cpDNA molecules) of these atypical molecules. The functionality of these atypical molecules is not known. However, because of the high copy number of complete chloroplast DNA molecules, these rare molecules may not exist at the levels affecting gene expression.

The multimeric and atypical molecules may result from recombinational events or the random cleavage and fusion of replication intermediates. Recombination between IRs carried on two separate monomers results in a dimer (Kolodner and Tewari 1979); subsequent recombination events between molecules presumably produces multimers. EM studies of chloroplast DNAs from plants with the normal pair of IRs revealed both head-to-head and head-to-tail dimers, with the former predominating (Kolodner and Tewari 1979). However, our analyses revealed a predominance of head-to-tail dimers (see the dimer molecules shown in Figures 3C, 5A, and 5B). Intramolecular recombination between the inverted repeats within a monomer only shifts the polarity of flanking single-copy regions, and the overall structure of the molecule is retained (Kolodner and Tewari 1979). However, recombination between repeats in the same direction within a multimeric molecule could produce separate circular molecules of aberrant sizes with various numbers of IRs, as revealed by our cytogenomic analyses (Figure 5D). It is also possible that the multimeric and aberrantly sized molecules were produced by random cleavage of long linear molecules, followed by fusion of ends, after rolling-circle replication.

Members of the Fabaceae are unique among angiosperms in that the chloroplast genome has only one copy of the sequences corresponding to the IRs. Previous studies have compared the physical structure of cpDNA between pea and species possessing IRs. Kolodner and Tewari (1979) demonstrated that pea possesses primarily head-to-tail dimers. Bendich and Smith (1990) observed that PFGE of pea cpDNA showed no bands larger than trimer size, whereas watermelon cpDNA possessed a tetrameric band. UV light microscopy of ethidium bromide–stained PFGE bands excised from the gel showed that pea cpDNA exists in circular form and not in rosette or catenated form (Bendich, 1991). Both studies concluded that the prominent bands contain primarily linear DNA molecules and that the majority of circular molecules remained in the well. Fiber-FISH revealed only monomeric and dimeric pea cpDNA molecules (Figures 1 and 3F), with only 25% of the total pea cpDNA molecules in circular form. It has been shown that the absence of one of the IRs is correlated with a rearranged chloroplast genome, as compared with species possessing the IRs (Palmer and Thompson, 1982). The presence of IRs may allow for the formation of higher order multimeric molecules, as revealed in both Arabidopsis and tobacco.

Cytogenomics allows for powerful analysis of rare events that cannot be detected by more traditional techniques, such as DNA gel blot hybridizations or the polymerase chain reaction. Using the cytogenomic approach, we revealed previously unknown structures and undocumented plasticity within a genome believed to be highly stable. This approach will be useful for detailed analysis of transgenic plants carrying an engineered chloroplast genome. The benefits of chloroplast transformation for high expression levels of transgenes and transgene containment are becoming increasingly important (Daniell et al., 1998). Cytogenomics will allow for the rapid establishment of transgene heteroplasmy at different developmental stages and the percentage of transgenic chloroplast genomes required for acceptable expression levels. Putative replication intermediates may be confirmed in the future with further improvement of the current technique, allowing detailed studies into the replication not only of plant organellar DNAs but of any nucleic acid using a light microscope.

METHODS

Plastid Isolation

Three- to 4-week-old plants of tobacco (*Nicotiana tabacum* cv Samsun), *Arabidopsis thaliana* ecotype Columbia (Col-0), and garden pea (*Pisum sativum*) were used for plastid isolation. Before chloroplast isolation, plants were placed in the dark for 72 hr. Leaves were harvested, placed on ice, washed in water, and blotted dry. All subsequent steps were performed at 4°C unless noted otherwise. Fifty grams of leaf tissue was homogenized in ice-cold grinding buffer (0.45 M sorbitol, 50 mM Tris, pH 7.6, 5 mM EDTA, 0.2% [w/v] BSA, 1.0% polyvinylpyrrolidone-360 [Sigma], 0.025% spermine, 0.025% spermidine, and 1 mL of β-mercaptoethanol). Slurry was filtered through

four layers of cheesecloth and two layers of Miracloth. Filtrate was centrifuged at 4000*g* to pellet chloroplasts. Chloroplasts were resuspended in 1 × wash buffer (0.35 M sorbitol, 50 mM Tris, pH 7.6, and 0.5% BSA). Centrifugation was repeated, and crude chloroplast pellet was resuspended in 36 mL of wash buffer. $MgCl_2$ was added to 10 mM, followed by DNase I (Sigma) at 25 μg/mL, and the chloroplast sample was incubated on ice for 0.5 hr. EDTA was then added to 50 mL, and chloroplasts were loaded onto Percoll density gradients (1:1 volume of 100% Percoll to 2 × wash buffer). Chloroplast gradients were centrifuged at 12,000*g* for 10 min in a swinging-bucket rotor (model JA-13; Beckman Instruments). The lower chloroplast band was removed and resuspended in 3 volumes of wash buffer plus 20 mM EDTA. Chloroplasts were pelleted at 3500*g* and resuspended in 2 mL of wash buffer plus 20 mM EDTA. Chloroplasts were mixed 1:1 with 50% glycerol and stored at −20°C for fluorescence in situ hybridization based on extended DNA fibers (fiber-FISH) experiments or embedded in agarose plugs for pulsed-field gel electrophoresis (PFGE).

PFGE Methods

Chloroplast suspensions were mixed 1:1 with 1% low-melting-point agarose (Gibco BRL) dissolved in wash buffer. Ninety microliters of organelle/agarose mixture was pipetted into plug molds (Bio-Rad) and allowed to solidify at 4°C. Plugs were placed into lysis buffer (2% sarkosyl, 0.45 M EDTA, and 10 μg/mL proteinase K) at 50°C, and buffer was exchanged three times in 30 hr. Plugs were washed in TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA) six times at 4°C, with the first two washes containing 1 mM phenylmethylsulfonyl fluoride (Sigma). Plugs were stored at 4°C. PFGE used a Chef Mapper III (Bio-Rad), 1% gels, and 0.5 × TBE buffer (1 × TBE buffer is 0.045 M Tris base, 0.045 M boric acid, and 0.001M EDTA, pH 8.0) cooled to 12°C. Details of PFGE conditions are given in the legend to Figure 1.

After PFGE, DNA was transferred to a Zetaprobe GT membrane (Bio-Rad), according to the manufacturer's directions. Thirty-six hours later, blots were dried under vacuum at 80°C for 1 hr. Labeling of probe DNA, membrane hybridization, and autoradiography were performed according to King et al. (1998).

Chloroplast DNA Visualization

DNA probes for FISH analyses were isolated using the Qiagen (Valencia, CA) midi-prep Tip-100 system. Fourteen plasmid clones containing all regions of the petunia chloroplast genome (Sytsma and Gottlieb, 1986) and three overlapping P1 chloroplast clones (MAB17, MCI3, and MAH2) from Arabidopsis were a gift from the Kazusa DNA Institute (Kisarazu, Chiba, Japan) (Sato et al., 1999).

Chloroplast DNA fibers were prepared on poly-L-lysine slides (Sigma). Five microliters of resuspended chloroplasts was placed in the center of a slide, and 15 μL of lysis buffer (2% sarkosyl, 0.25% SDS, 50 mM Tris, pH 7.4, 50 mM EDTA, pH 8.0, and 0.5% Triton X-100) was added. Slides were incubated for 10 min at 37°C, at which time a 22 × 40-mm cover slip was gently placed over the chloroplast suspension. DNA fibers were fixed at 60°C for 10 min, and cover slips were removed in a 3:1 solution of 100% ethanol:glacial acetic acid and dried at 60°C for 15 min. Probe labeling, fiber-FISH hybridization, detection, and image capture were as described previously (Jackson et al., 1999). Each experiment was performed with two independently isolated plastid samples and repeated a minimum of six times. Images of tobacco chloroplast DNA (cpDNAs) for size estimations were collected randomly from 12 slides. We confirmed the lack of nuclear DNA contamination based on the lack of signal from a nuclear (5S rDNA) clone hybridized to tobacco chloroplast fibers. For size estimations, regression analyses of fiber measurements were performed using Excel 2000 (Microsoft, Redmond, WA). Final image adjustments (brightness/contrast) were made with Photoshop 5.1 (Adobe Systems, Mountain View, CA).

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